Reference: *Biol. Bull.*, 153: 577–590. (December, 1977)

STUDIES ON THE ANNUAL REPRODUCTIVE CYCLE OF THE SEA URCHIN AND THE ACID PHOSPHATASE ACTIVITY OF RELICT OVA

REIKO MASUDA[1] AND JEAN C. DAN

Faculty of Science, Ochanomizu University, Bunkyo-ku, Tokyo 112; and Tateyama Marine Laboratory, Ochanomizu University, Tateyama-shi, Chiba 294-03, Japan

Among the echinoderms, in which the oocytes grow into mature eggs during a few months before the reproductive season, the ovaries show an annual cyclic change. Nuclear changes, cortical granule formation, vitellogenesis and RNA-synthesis in sea urchin oogenesis have been studied with the electron microscope (Millonig, Bosco and Gimbertone, 1968; Anderson, 1968; Vehrey and Moyer, 1967). A radial nerve factor has been found to induce spawning in echinoids (Cochran and Engelmann, 1972), and changes in the amount of 1-methyladenine in the sea urchin ovary during oogenesis have been reported (Kanatani, 1974). In spite of the important status of the sea urchin egg, there are several fundamental points in connection with the annual morphological changes of the sea urchin ovary that have not been made completely clear.

Recently, it has been found that the follicle cells of asteroidean ovaries produce 1-MA during the process of oocyte maturation (Hirai, Chida and Kanatani, 1973); but in the Echinoidea, it is suggested that there may be a dynamic relationship between the germ cells and nongerm cells, which are variously known as follicle cells, accessory cells and nutritive phagocytes (Holland and Giese, 1965; Chatlynne, 1969; Bal, 1970). The annual changes that take place in the accessory cells are poorly understood, and the function of the accessory cells is still under discussion (Takashima and Tominaga, 1975).

This study focuses on the accessory cells of *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* during the ovarian cycle, using the staging concept of Fugi (1960): stage I (spent recovering stage); stage II (growing stage); stage III (premature stage); stage IV (mature stage); and stage V (spent stage). The acid phosphatase activity of relict (unshed) ova at stage V is also observed, and a model is proposed to account for the process by which the relict ova degenerate.

Materials and Methods

Two species of sea urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus,* were collected once or twice a month from May, 1971 to 1973, along the coast near the Tateyama Marine Laboratory. Ovaries were removed and tissue samples were fixed with 1% OsO_4 in sea water for one hour at room temperature. After dehydration, the samples were embedded in Epon 812. Half-micron sections were cut on an ultramicrotome, stained with 0.5% toluidine blue in phosphate buffer (pH 7.0) for 15 min at room temperature and observed by light microscopy.

[1] Present address: Department of Anatomy, Tokyo Women's Medical College, Shinjuku-ku, Tokyo 162, Japan.

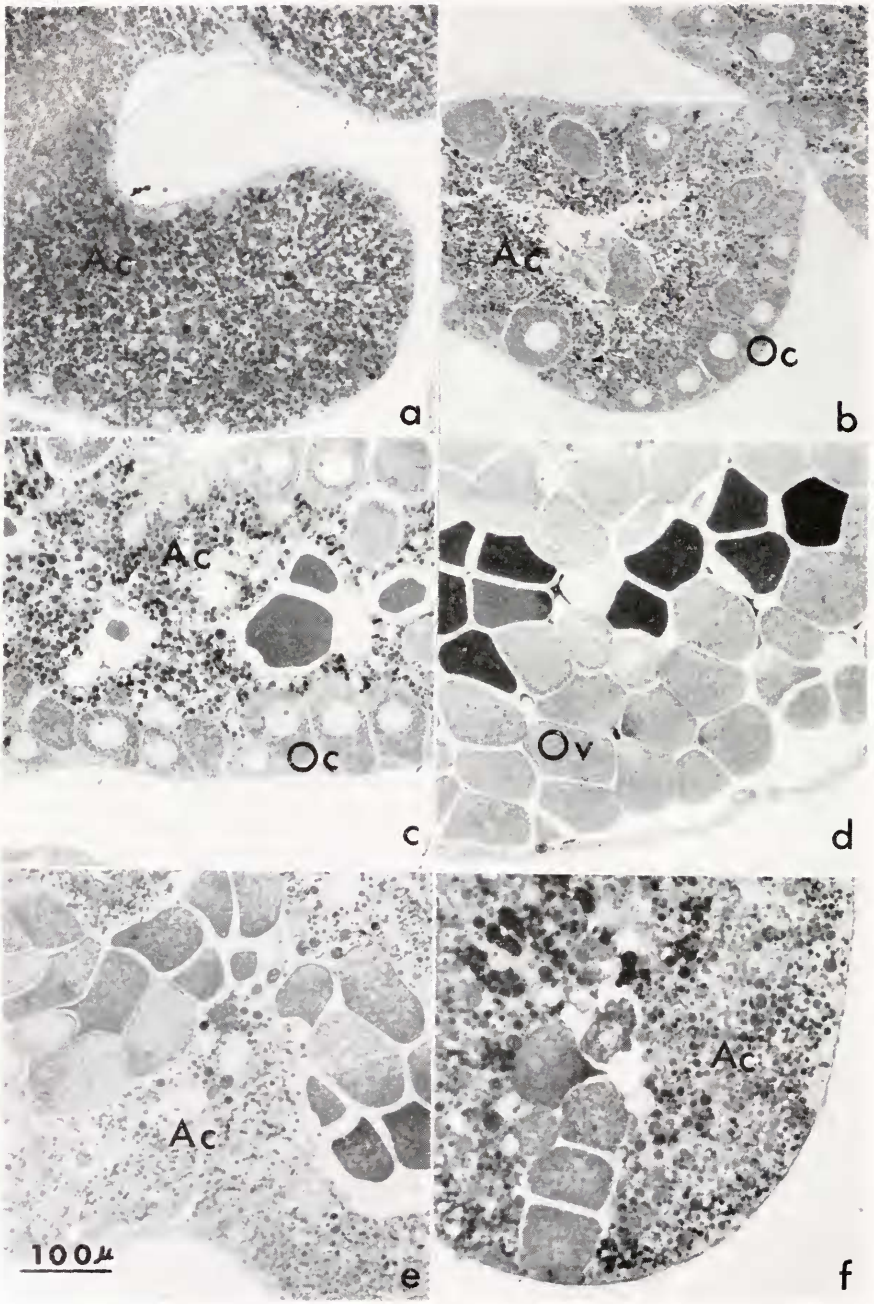

FIGURE 1. Thick sections at various stages of Epon-embedded ovaries of *Anthocidaris crassispina*, stained with toluidine blue: a, stage I, January—accessory cells including numerous globules occupy the ovariole; b, stage II, March—many growing oocytes are attached to the ovarian wall; c, stage III, June—fully grown oocytes complete meiosis; d, stage IV, August—

The gonad indices were also determined for these two species (gonad wet weight/body weight × 100). About ten samples were collected for each average index value from March, 1972 to September, 1973. Sea surface temperature data from Ninomiya and Yorogami (1974) were used.

Acid phosphatase activity was investigated, using *Anthocidaris crassispina* at stage V (spent stage). Eggs mixed with some accessory cells were spawned into sea water following introduction of 0.5 M KCl into the body cavity, and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 min. Samples were incubated at 38° C for one hour in Gomori's solution [0.05 M acetate buffer (pH 5.0) 500 ml, lead nitrate 0.6 g, 3% glycerophosphate 50.0 ml], followed by washing in deionized water containing 1% yellow ammonium sulfide. Samples were embedded in Epon 812, and thick sections (0.5 μm) were made for observation with the light microscope. As control, Gomori solution without substrate (glycerophosphate) was used as the incubation medium. The same procedure was also carried out with ovaries in stage III (premature stage).

Specimens were fixed for electron microscopy with 2.5% glutaraldehyde for one hour, post-fixed for one hour with 1% OsO_4 in 0.1 M phosphate buffer, and embedded in Epon 812. Thin sections were stained with uranyl acetate and observed with an Hitachi HS-7D electron microscope.

Results

Ovarian cycle

Figure 1 shows a cross-section of *Anthocidaris crassispina* ovarioles. From December to March, the main part of the stage I ovary is filled with accessory cells containing numerous globules, some of them 10 μm–15 μm in diameter. Small oocytes about 10 μm in diameter are observed along the ovarian wall (Fig. 1a). From March to May (stage II), the growing oocytes (Fig. 1b) rapidly come to contain many yolk granules of various sizes. During May and June (stage III), the full-sized oocytes undergo the reduction divisions and move toward the center of the ovariole to be stored as mature ova until they are spawned (Fig. 1c). As the reproductive season begins, the stage IV ovariole is almost completely filled with ova and a very few accessory cells can be observed along the ovarian wall (Fig. 1d). After the end of the spawning season (stage V), the amount of space occupied by accessory cells increases, and the ovary at this stage sometimes contains unshed (relict) ova which are in the process of degenerating (Fig. 1e, f).

The same morphological changes are observed in *Hemicentrotus pulcherrimus*. From May to November (stage I), the ovarioles are filled with accessory cells containing globules (Fig. 2a). The small oocytes arranged along the ovarian wall grow rapidly in November to full size (Fig. 2b, stage II). Globules are less conspicuous in the accessory cells (Fig. 2c, stage III), and the area occupied by each cell seems to be smaller than before. The gonad index is highest at this stage (Fig. 3). Sometimes "empty" accessory cells are observed around fully

mature eggs occupy most of the lumen and flattened accessory cells line the ovarian wall; e, stage V, September—relict ova are present in the center of the ovariole, and the space occupied by accessory cells has increased; and f, stage V, December—relict ova degenerate at the center of the ovarian lumen. Ac represents the area of accessory cells; Oc, oocyte; and Ov, ovum.

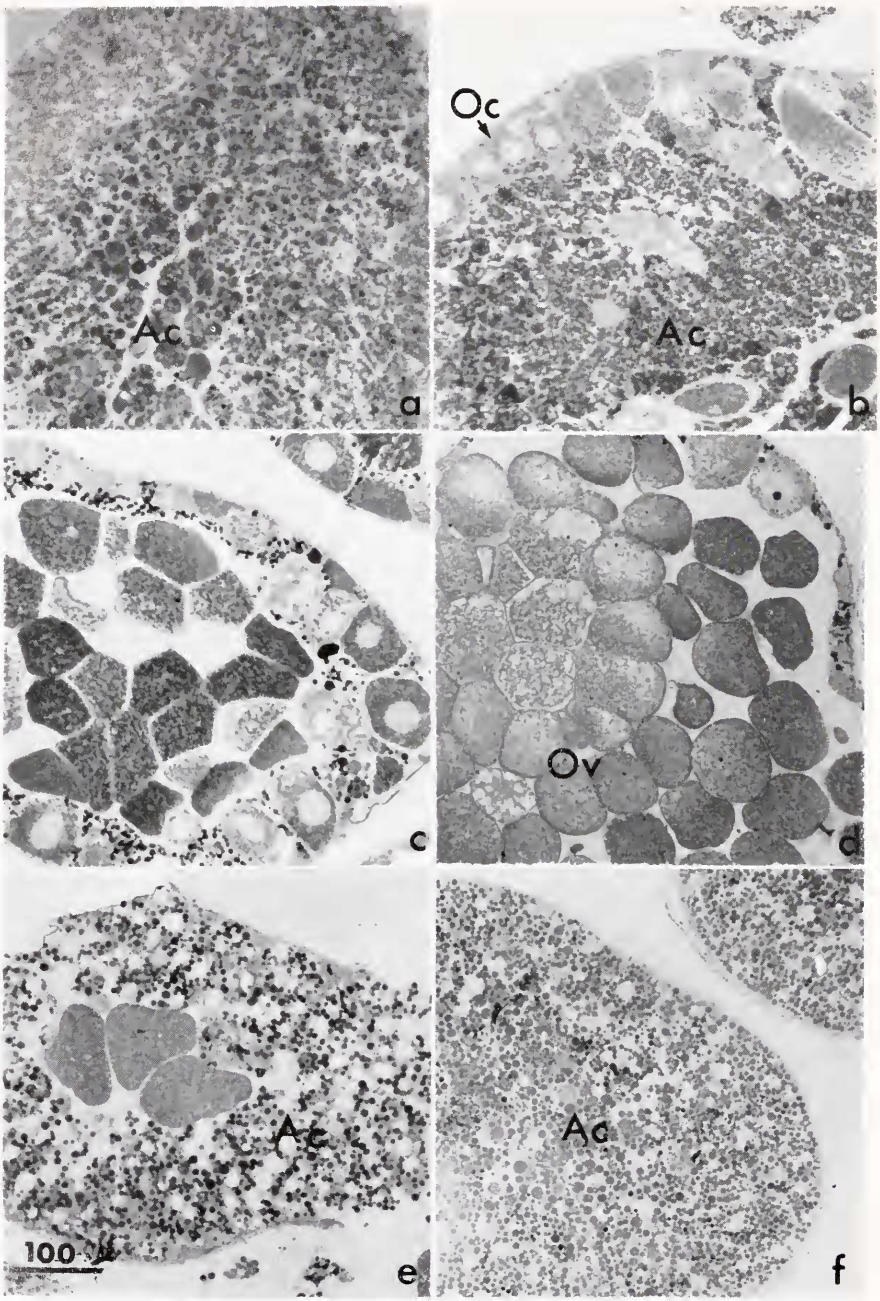

FIGURE 2. Thick sections of Epon-embedded ovariole of *Hemicentrotus pulcherrimus:* a, stage I, November—numerous accessory cells occupy the lumen and a few small oocytes are present along the wall; b, stage II, December—fully grown oocytes are seen along the wall; c, late state of stage III, January—fully grown oocytes complete meiosis, mature eggs occupy the

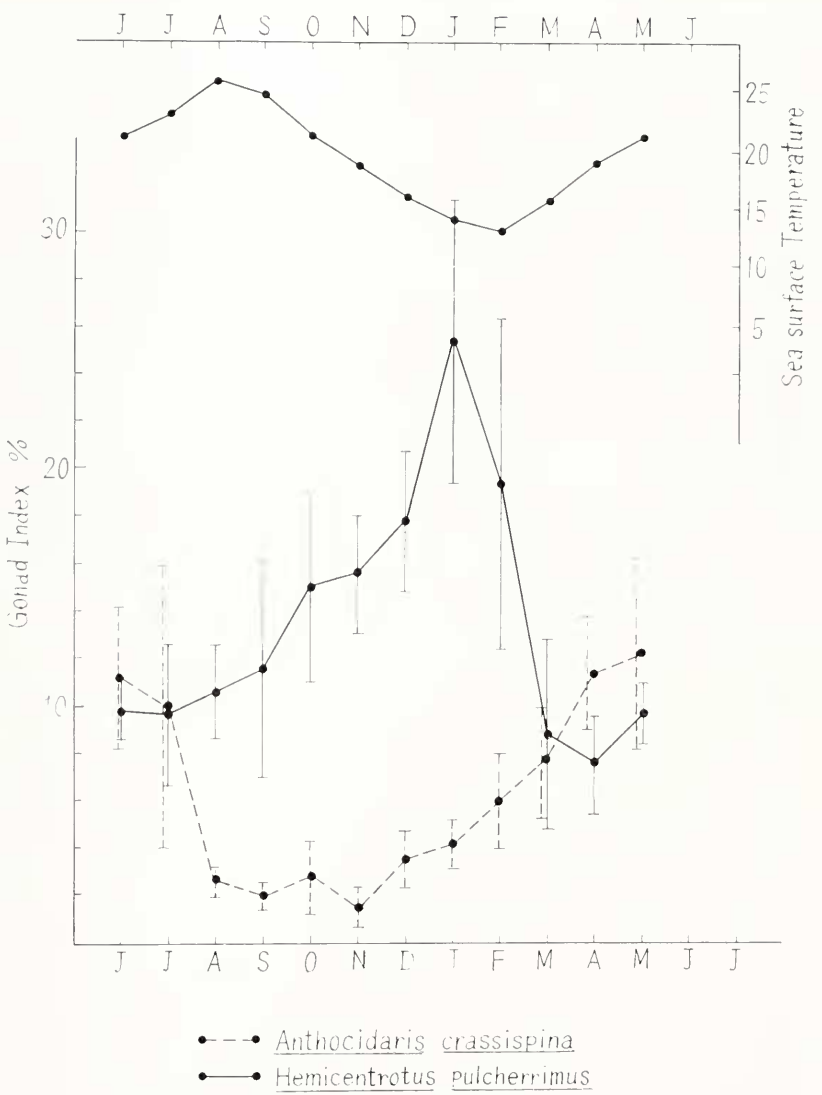

FIGURE 3. The gonad index (gonad wet weight/body temperature × 100) and sea surface temperature. Data were taken on the day of collection.

grown oocytes. Most of these oocytes complete meiosis during early December, and the mature eggs are stored in the center of each ovariole. The reproductive season (stage IV, Fig. 2d) begins in January and ends in March. Mature eggs

lumen and a few oocytes are present along the wall; d, stage IV, February—mature eggs in the lumen, some of which are degenerating; e, stage V, April—a few relict ova are present in the center of lumen, with more accessory cells present than in the previous stage; and f, stage I, May—the same stage as Figure 2a.

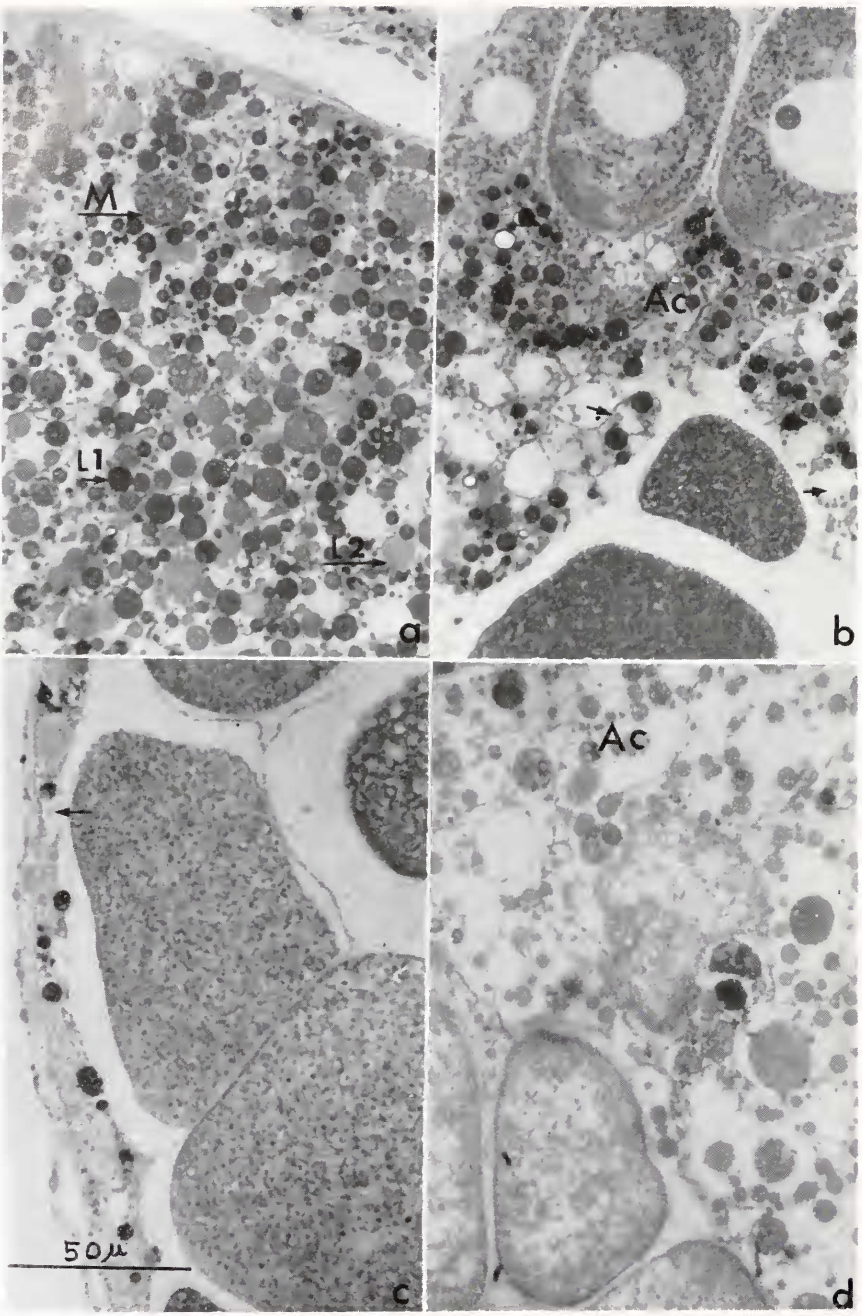

FIGURE 4. Various changes in the inclusions of the accessory cells in the course of the ovarian cycle in *A. crassispina*: a, stage I—accessory cell is filled with various types of globules (L1, large globule 1; L2, large globule 2; M, mosaic globule); b, stage III—empty cells

occupy the whole space within the ovary, and only a small number of "empty," flattened accessory cells can be observed near the end of the reproductive season; the relict ova remaining in the center of the ovary become vacuolated (Fig. 4d). After the reproductive season (stage V), the ovarioles are again filled with globules containing accessory cells (Fig. 2e). The layer of accessory cells gradually becomes thicker, until finally the ovarioles are completely occupied by accessory cells containing globules of various sizes. The relict ova have all degenerated by this stage (Fig. 2f).

On the basis of these observations, the annual ovarian changes of two species of sea urchins at Tateyama have been summarized following the staging of Fuji (1960). Of these species, *H. pulcherrimus* has the longer period assignable to stage I, but their ovarian cycles follow basically the same pattern, in which the gonad index (Fig. 3) shows the highest value at stage III (*H. pulcherrimus* in January, *A. crassispina* in the middle of May). The mean value of the sea surface temperature varies from 13.1° C in February to 25.9° C in August.

In *Strongylocentrotus purpuratus*, Holland and Giese (1965) reported that oogonial clusters could not be found during the winter (stages III and IV), while Chatlynne (1969) described that oogonia are very difficult to find in the recovering spent stage (stage I) but are numerous in the winter (stages III and V). According to Gonor (1973a), oogonial clusters were found at all times of the year, but from November through March (stages III and V), their numbers are low and they are easily overlooked. In the present study, the clusters of germ cells including oogonia and small oocytes were observed in *A. crassispina* at stage I, while in *H. pulcherrimus*, these cells were very difficult to observe at the same stage. The germ cells at stage I in *H. pulcherrimus* seem to be dormant.

Accessory cells

At stage I, the ovarioles are filled with accessory cells containing conspicuous globules, which can be divided into three types: large globule 1, which stains strongly with toluidine blue; large globule 2, which stains weakly with toluidine blue; and mosaic globule, which contains cortical granule-like structures. The diameters of these globules range from 3 μm–10 μm (Fig. 4a). At stage III, many "empty" accessory cells could be observed around fully grown oocytes and ova (Fig. 4b, arrow). At stage IV, the maturing stage, accessory cells are seen along the ovarian wall, and the center of the ovarioles is filled with mature eggs (Fig. 4c). After the reproductive season, the space occupied by accessory cells increases, and a few relict ova are seen at the center of the lumen (Fig. 4d). Takashima (1968) divided these globules into four types; large granule A, large granule B, large granule C and oil droplets. The large granule A corresponds to mosaic globules in the present study, large granule B to large globule 2 and large granule C to large globule 1.

Figure 5 shows a cross-section of an ovariole at stage V. At this time, mosaic globules become conspicuous in the accessory cells surrounding the degenerating

(arrows) are seen around full-sized oocytes or ova; c, stage IV—flat "empty" accessory cells line the ovarian wall (arrow); and d, stage V—"relict" ova in which cytoplasm but not cortex appears disrupted. Accessory cells have fewer globules.

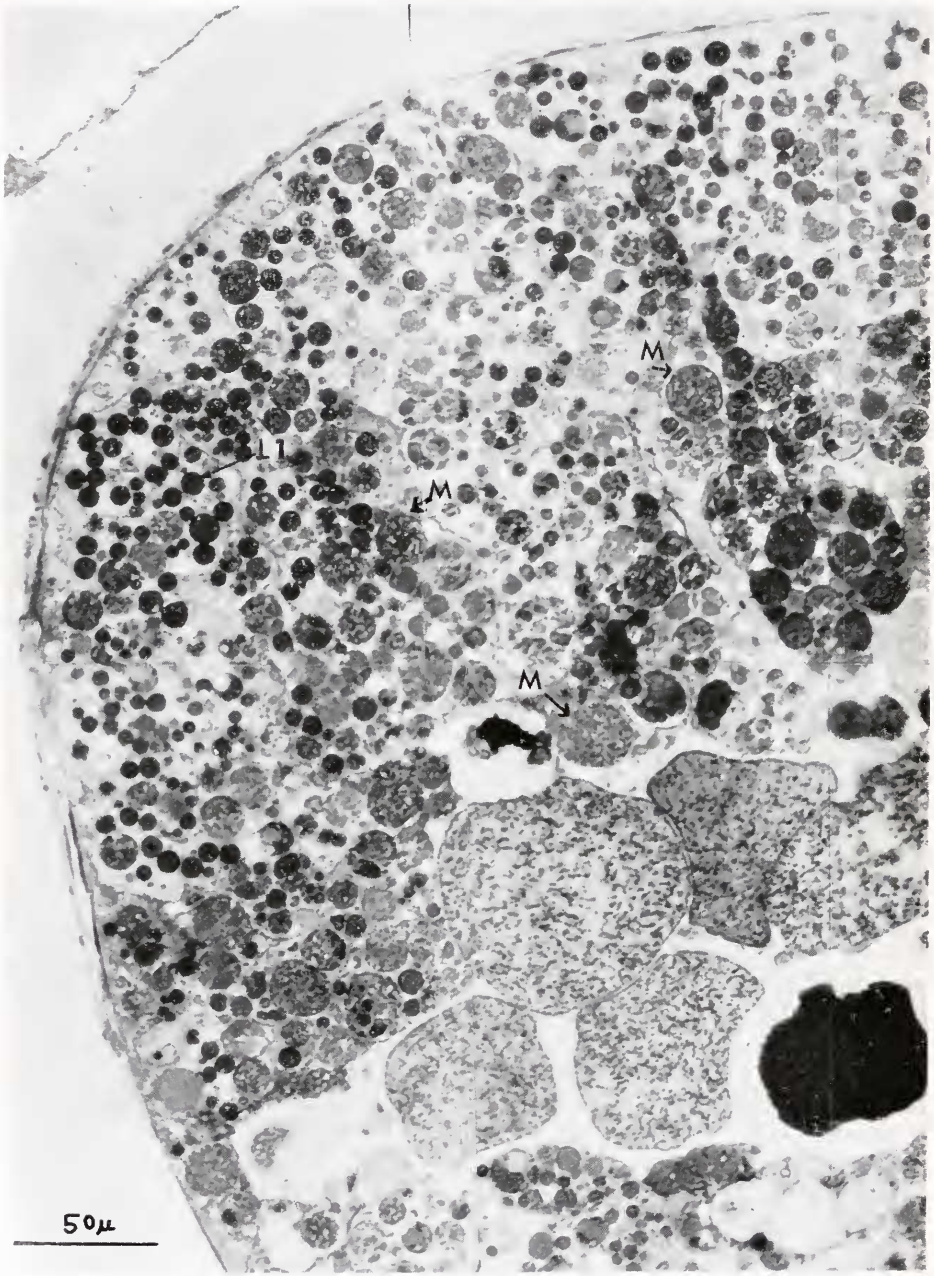

FIGURE 5. Cross-section of an ovariole of *A. crassispina* at stage V (December). Unshed (relict) ova are seen at the center of the lumen, surrounded by many accessory cells containing mosaic globules (L1, large globule 1; M, mosaic globule).

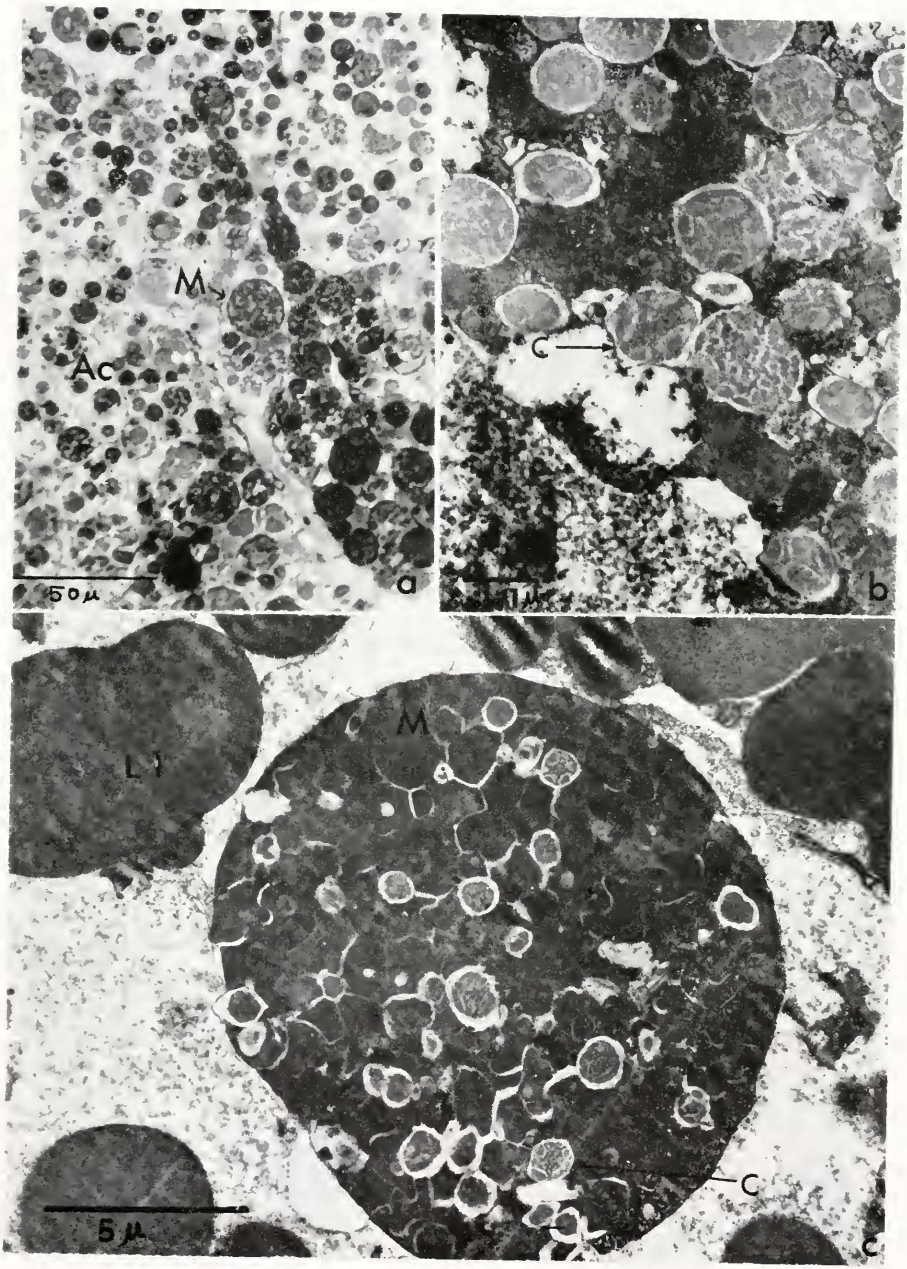

FIGURE 6. Fine structure of mosaic globule: a, light microscopic observation of mosaic globules; b, c, electron microscopic observations of mosaic globules, containing cortical granule-like structures. Ac represents accessory cell; c, cortical granule-like structure; LI, large globule; and M, mosaic globule.

ova, the size of which is 10 μm–30 μm, and the two types of large globules, large globule 1 and large globule 2, are rarely seen compared with the state of stage I (Fig. 4a). The mosaic globules aways contain structures which are about 1 μm in diameter and have the appearance of cortical granules (Fig. 6a, b, c).

Acid phosphatase activity of relict ova

In both species, the relict ova remaining in the ovarioles at stage V undergo degenerative changes: large "empty" spaces are present in the cytoplasm, and the number of yolk granules is greatly reduced (Fig. 1f, 2d, 4d, 5); or the shapes of the cells depart markedly from the spherical (Fig. 1e, 2f, 4d). In most cases, however, the egg cortex seems to remain relatively unchanged (Fig. 4d, 5). Eggs showing these characteristics were collected and subjected to a Gomori test to detect acid phosphatase activity. Deposits of lead sulfate are observed in the ova, accessory cells (Fig. 7a) and in small oocytes (Fig. 7b). Specimens which were incubated without substrate do not show such deposits (Fig. 7c, control). In some cases (Fig. 7a, b), the phosphatase activity is localized in an irregular manner. When the same procedures were carried out with eggs collected during the prematuring stage (stage III), the acid phosphatase activity was found to be about the same as that of the control (Fig. 7d, e; control).

These results show that relict ova have strong acid phosphatase activity compared to normal ova.

Discussion

Fluctuations of the food supply may explain the great variability observed in gonad size and fertility; a sufficient food supply in the gonad permits a large number of oocytes to become ova simultaneously (Boolootian, 1966). Gonor (1973a) studied the influence of environmental factors in the annual changes of the ovary. The gonad indices of *A. crassispina* and *H. pulcherrimus* collected at the coast of Tateyama (Tokyo Bay) are alike in showing a steady increase in the size of the ovary during the period of oocyte growth preceding each breeding season, but they differ conspicuously in the minimum size observed after the end of spawning. The *A. crassispina* ovary diminishes to 15% of its maximum size, as spawning ends in late summer, and remains very small through the autumn until December. The *H. pulcherrimus* ovary retains 33% of its maximum weight at the end of the spawning season in April, and immediately begins to increase slowly in late spring and summer and then rapidly through the autumn, until November (*i.e.*, the ovary is in stage I during this time when it acquires 50% of its maximum bulk). This increase of gonad weight in spring, summer and autumn means that the gonad stores nutrients and prepares for the oogenesis which will take place in December when the food supply may be insufficient. Thus, from April until November, it might be more accurate to regard the *H. pulcherrimus* ovary as a nutrient-storing, rather than a gamete-producing, organ. Gonor (1973b) observed the ovary of *S. purpuratus* and divided the annual changes into two phases: first, the period of oogonial proliferation, resulting in an increase in the number of clusters; and secondly, the period when the oogonia in clusters transform into

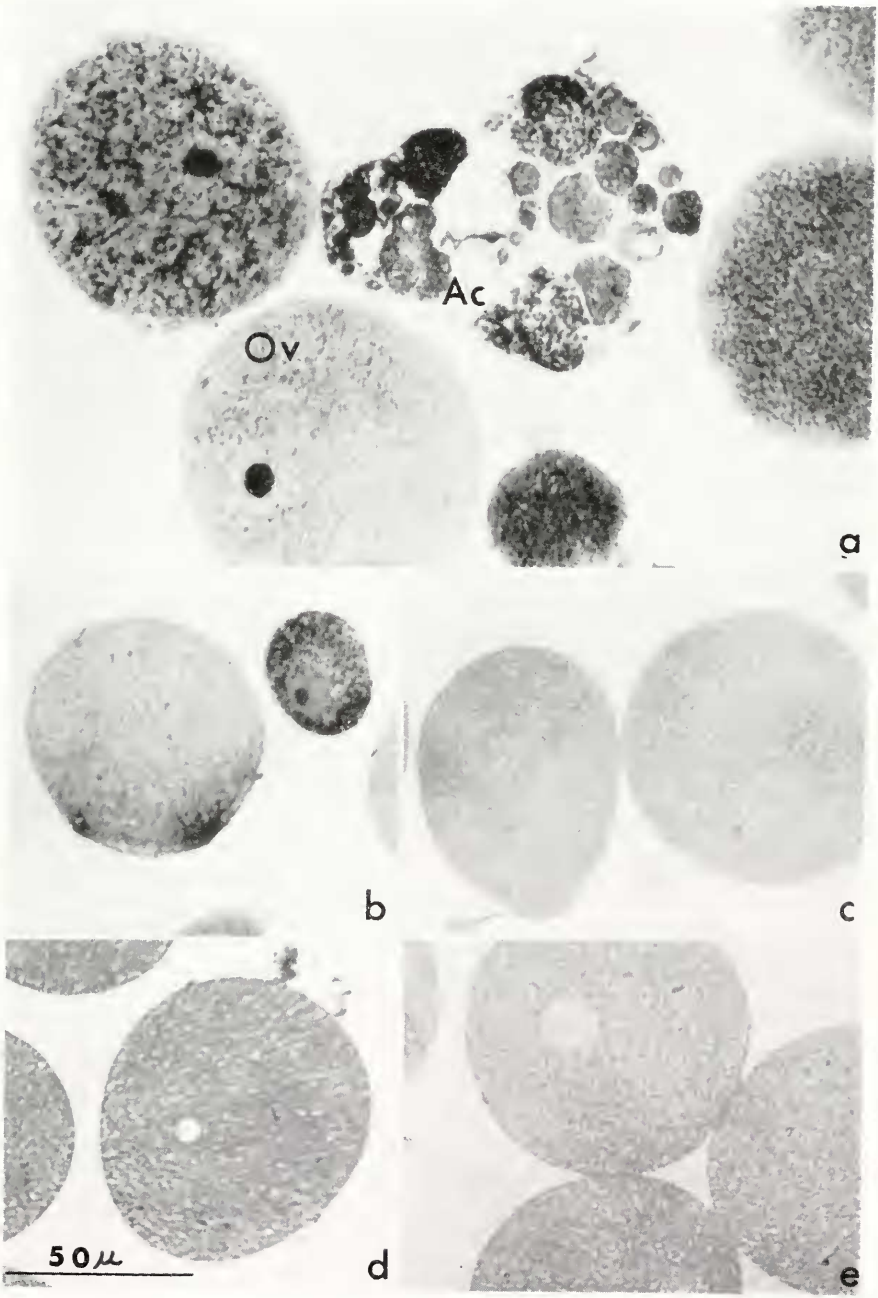

FIGURE 7. Relict ova and mature ova of *A. crassispina* treated by Gomori method: a, accessory cells and relict ova at stage V; b, relict ovum and small oocyte; c, control (without substrate) at stage V; d, mature ova at stage IV; and e, control (without substrate) at stage IV. Ac represents accessory cells; and Ov, ovum.

primary oocytes and become ova. This first period corresponds to the stage when nutrients are stored.

Many observers of the sea urchin gonad have reported on the accessory cells. In one state, they appear almost empty except for a few inconspicuous globules (Chatlynne, 1969). The two species of sea urchins used in this study showed a similar pattern of changing. In particular, the accessory cells around the degenerating ova contain numerous mosaic globules (Fig. 5) which include cortical granule-like structures (Fig. 6). This observation presents an interesting problem in connection with the mode of disposal of the relict ova.

The globules in the accessory cells are stained very strongly by the periodic-acid-Schiff technique, indicating that they include substances which may be of nutritional value for the developing oocytes (Cowden, 1962; Chatlynne, 1969; Bal, 1970). So far there is only indirect evidence that these substances are transferred to the oocytes; *e.g.,* Takashima and Tominaga (1975) found that ^{3}H-D-glucose is present in "nurse cells" three days after injection and only appears in the oocytes after the fourteenth day. Also, a number of investigators have observed that the space occupied by accessory cells decreases as the sensitivity of the oocytes to the PAS reaction becomes stronger, until only empty accessory cells are observed around the fully grown oocytes. The mechanism by which the nutrients are transferred from accessory cells to oocytes has been an object of concern on the part of many investigators. Liebman (1950) observed figures which he interpreted as oocytes phagocytizing accessory cells. Bal (1970) reported that glycogen particles, packed with a protein-like substance, are released into the intercellular spaces. Takashima and Takashima (1965), Tsukahara (1970) and Bal (1970) presented electron microscopical evidence that glycogen particles are taken into the oocytes as pinosomes and used as yolk precursor material.

The manner in which unshed sea urchin gametes are disposed after the end of the breeding season seems to have received only casual attention. Fuji (1960) proposed that "unshed" degenerating ova are absorbed into the follicle cells. Holland and Giese (1965) observed that spermatids are injested by the nutritive phagocytes of the testis early in the reproductive season and again at its end, and assumed that unshed ova also are disposed by a similar process in the ovary. These investigators, however, do not show intermediate stages of relict ova being phagocytized, although they suggest that this might take place. Beig and Cruz-Landim (1975) have investigated in some detail the process by which the residual germ cells are removed from the sea urchin testis and report that spermatids and spermatozoa are phagocytized by "nurse cells" during the interval between reproductive periods. They show electron micrographs in which sperm nuclei, and in some cases flagella, are contained within "digestive vacuoles" *ca.* 10 μm in diameter. Both the size of these vacuoles and the fact that they include left-over gamete organelles suggest a functional parallel with the "mosaic globules" of the sea urchin ovary described in this study. While it is easy to imagine spermatids and spermatozoa being injested by interstitial cells of the testis, the phagocytosis of relict eggs would seem to present a more difficult problem to the accessory cells of the ovary. Several observations made in the course of the present study indicate that autolytic activity may be responsible for the degenerative changes observed in the relict ova. The results of the Gomori test show a significantly

stronger acid phosphatase activity in these cells than in normal ova. The number of yolk granules in the degenerating ova is greatly reduced, and their shape is irregular. The cortical layer remains relatively unchanged as compared with normal ova. These morphological observations, and the results of Gomori tests, suggest that lysosomal disruption might occur during the course of degeneration of sea urchin ova. After this stage, "relict" ova might be taken into accessory cells as in the case of spermatids (Holland and Giese, 1965; Beig and Cruz-Landim, 1975). Numerous mosaic globules appear around degenerating ova at stage V, which might indicate some intimate relationship between mosaic globules and degenerating ova. The result that some of the accessory cells and small oocytes showed marked deposits of lead sulfate suggests that these cells degenerate by autolytic processes. Further detailed observations are necessary to make this process completely clear.

Summary

1. Specimens of the sea urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus*, were collected each month, their ovaries were fixed and embedded in Epon 812, and thick sections were observed with the light microscope.

2. The sea urchin ovary has a clearly defined state in which nutrient globules are produced and stored in accessory cells. Especially in *Hemicentrotus pulcherrimus*, this state continued from May to November. The number of globules in the accessory cells fluctuated with the course of the reproductive cycle.

3. Toward the end of the breeding season, strong acid phosphatase activity was detected in the unshed (relict) ova as they degenerated, and numerous large mosaic globules containing cortical granules appeared in the accessory cells. It is proposed that the cytoplasm of relict ova is disrupted by the activity of their lysosomes.

Literature Cited

Anderson, E., 1968. Oocyte differentiation in the sea urchin *Arbacia punctulata*, with particular reference to the origin of cortical granules and their participation in the cortical reaction. *J. Cell Biol.*, **37**: 514–539.

Bal, A. K., 1970. Ultrastructural changes in the accessory cells and the oocyte surface of the sea urchin *Strongylocentrotus drobachiensis* during vitellogenesis. *Z. Zellforsh Microsc. Anat.*, **111**: 1–14.

Beig, D., and C. da Cruz-Landim, 1975. Sperm reabsorption in sea urchin (*Echinodermetra lacunter*). *Ciencia Cultura*, **27**: 221–228.

Boolootian, R. A., 1966. Reproductive physiology, Pages 561–613 in R. A. Boolootian, Ed., *Physiology of Echinodermata*. Interscience Publishers, New York.

Chatlynne, L. G., 1969. A histochemical study of oogenesis in the sea urchin *Strongylocentrotus purpuratus*. *Biol. Bull.*, **136**: 167–184.

Cochran, R. C., and F. Engelmann, 1972. Echinoid spawning induced by a radial nerve factor. *Science*, **178**: 423–424.

Cowden, R. R., 1962. RNA and yolk synthesis in growing oocytes of the sea urchin *Lytechinus variegatus*. *Exp. Cell Res.*, **28**: 600–609.

Fuji, A., 1960. Studies of the biology of the sea urchin I. Superficial and histological gonadal changes in the gametogenic process of two sea urchins, *Strongylocentrotus nudus* and *S. intermedius*. *Bull. Fac. Fish. Hokkaido Univ.*, **11**: 1–14.

Gonor, J. J., 1973a. Reproductive cycles in Oregon populations of the echinoid, *Strongylocen-*

trotus purpuratus. I. Annual gonad growth and ovarian gametogenic cycles. *J. Exp. Mar. Biol. Ecol.*, **12**: 45–64.

Gonor, J. J., 1973b. Reproductive cycles in Oregon populations of the echinoid, *Strongylocentrotus purpuratus*. II. Seasonal changes in oocyte growth and in abundance of gametogenic stages in the ovary. *J. Exp. Mar. Biol. Ecol.*, **12**: 65–78.

Hirai, S., K. Chida, and H. Kanatani, 1973. Role of follicle cells in maturation of starfish oocyte. *Dev. Growth Differ.*, **15**: 21–31.

Holland, N. D., and A. C. Giese, 1965. An autoradiographic investigation of the gonads of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.*, **128**: 241–258.

Kanatani, H., 1974. Presence of 1-methladenine in sea urchin gonad and its relation to oocyte maturation. *Dev. Growth Differ.*, **16**: 159–170.

Liebman, E., 1950. The leukocytes of *Arbacia punctulata*. *Biol. Bull.*, **98**: 46–59.

Millonig, G., G. Bosco, and L. Gimbertone, 1968. Fine structure analysis of oogenesis in sea urchins. *J. Exp. Zool.*, **169**: 239–314.

Ninomiya, T., and S. Yorogami, 1974. Studies on the sea surface temperatures along the coasts. *Bull. Chiba-ken Fisheries Lab.*, **33**: 39–50 (in Japanese).

Takashima, E., and Y. Takashima, 1965. Studies on the submicroscopical structures of the nurse cells in sea urchin ovary, with special reference to glycogen particles. *Okajimas Folia Anat. Jpn.*, **40**: 819–831.

Takashima, Y., 1968. Electron microscopic observation on the nurse cells in sea urchin ovary. *Med. J. Osaka Univ.* **19**: 113–126.

Takashima, Y., and A. Tominaga, 1975. Ultrastructure and behavior of giant granules in nurse cells in sea urchin ovary. *Int. Cong. Anat. Tokyo*, **10**: 455.

Tsukahara, J., 1970. Formation and behavior of pinosomes in the sea urchin oocyte during oogenesis. *Dev. Growth Differ.*, **12**: 53–64.

Vehrey, C. A., and F. H. Moyer, 1967. Fine structural changes during sea urchin oogenesis. *J. Exp. Zool.*, **164**: 195–207.